 DOI: 10.24425/academiaPAS.2024.152930

A Factory of Bacterial Weaponry

Understanding one of the key virulence mechanisms of *Porphyromonas gingivalis* bacteria could lead to improved methods for preventing and treating periodontitis and related diseases.

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The human body hosts a diverse array of bacteria that together form its *microbiome*. These bacteria provide essential nutrients, aid in digestion, and help protect against infections. However, under certain unfavorable conditions – such as poor health, an imbalanced diet, substance use, stress, aging, or genetic factors – the composition of the microbiome can change. When this happens, bacteria that are typically harmless components of a healthy microbiome can become pathogenic. They may partially replace beneficial bacteria, contributing to the development of diseases.

The microbiome of the human body is made up of a variety of specialized microbiomes. The oral microbiome, found in the oral cavity, is one of the most diverse. Disruptions within it can lead to diseases affecting the tissues around the teeth, such as periodontitis, commonly known as gum disease. During the progression of periodontitis, a biofilm – a multi-layered structure made up of bacterial cells and their secretions – forms below the gumline. This biofilm includes many bacterial species, with *Porphyromonas gingivalis* being one of the most significant contributors to the disease.

P. gingivalis is an anaerobic bacterium with a structure that is typical of a certain group known as *Gram-negative bacteria*. These bacteria are often (though not always) covered by a sugar-based cap-

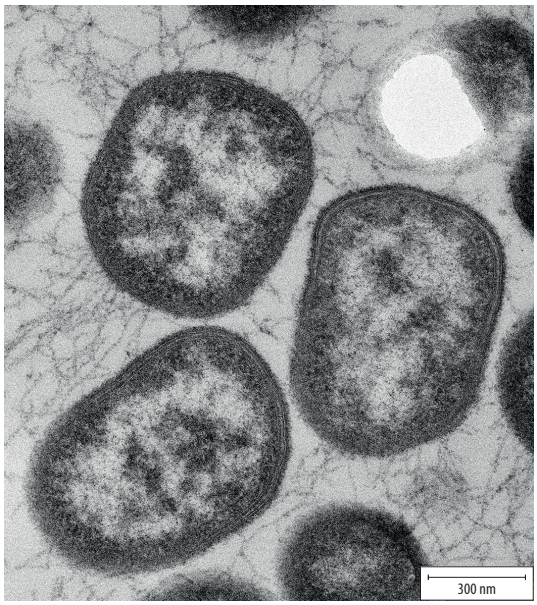

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Image of a *P. gingivalis* cell (strain ATCC 33277) captured using a transmission electron microscope. The thread-like structures visible between cells are fimbriae. The scale bar is shown in the bottom-right corner

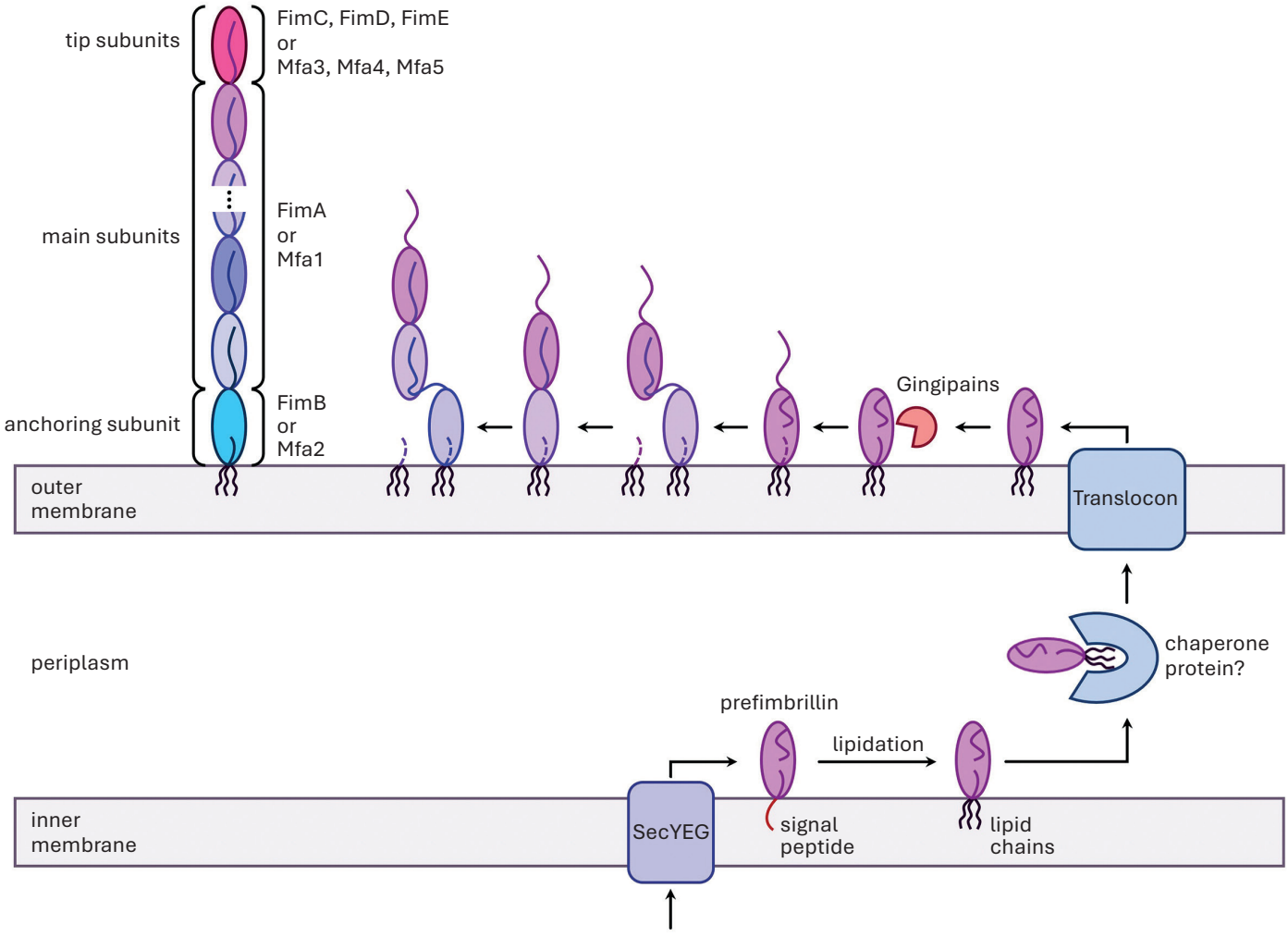

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Diagram of a cross-section of the *P. gingivalis* cell surface and the mechanism of fimbriae secretion and maturation by the studied complex (translokon)

sule. Beneath this capsule is an outer membrane, followed by a watery layer known as the *periplasm*, which contains the thin cell wall. The inner membrane surrounds the rest of the bacterial cell (the cytoplasm). The cell wall of Gram-negative bacteria is so thin that it does not retain the crystal violet dye used in the Gram-staining technique. In comparison, Gram-positive bacteria have thick cell wall that retain the dye but lack an outer membrane.

To be able to grow, *P. gingivalis* requires *peptides* – molecules derived from protein breakdown – and *heme*, an iron-containing molecule released from host proteins such as hemoglobin. Even in relatively small amounts, these bacteria can disrupt the human immune system, amplify inflammation, and destabilize the balance of the oral microbiome.

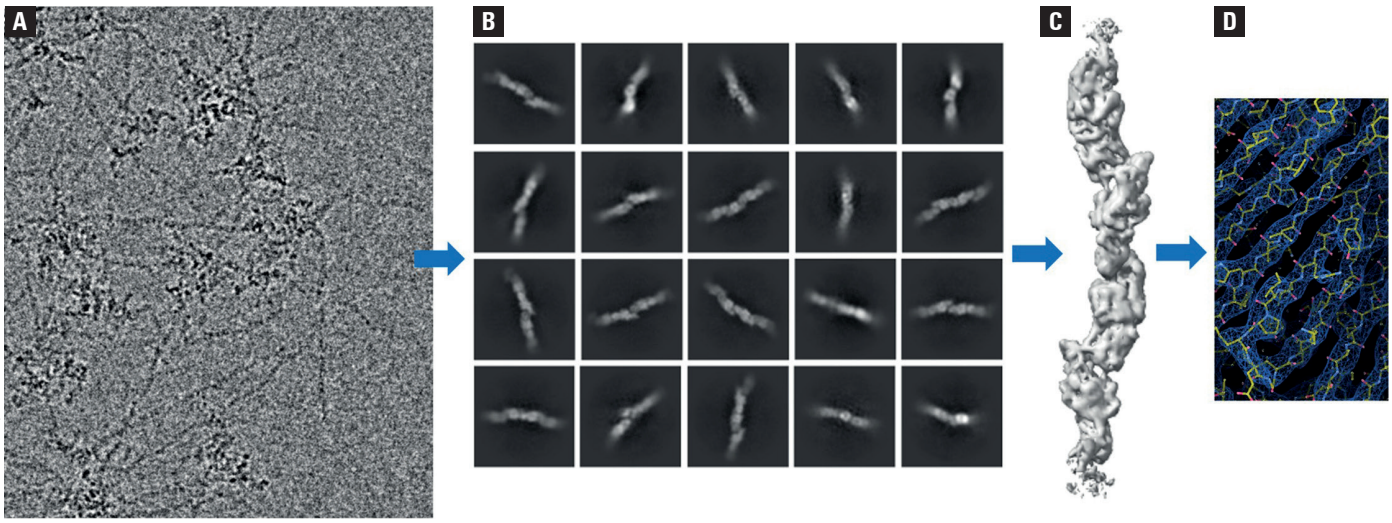
Periodontitis caused by *P. gingivalis* does not just result in gum recession, bone loss, and, in severe cases, tooth loss. The chronic inflammation associated with the disease can also spread to other parts of the body, contributing to various health conditions. Research from the past several decades has demonstrated strong links between periodontitis and an increased risk of diabetes, rheumatoid arthritis, aspiration pneumonia, cardiovascular diseases, certain cancers, and pregnancy complications. Additionally, biomolecules produced by this bacterium have been found in several regions of the brains of individuals with Alzheimer's disease. Understanding how this bacterium causes such widespread harm in the human body and uncovering the mechanisms it uses are crucial steps toward developing effective strategies to combat it.

A rich arsenal

P. gingivalis owes its great pathological potential to its diverse array of *virulence factors* – a term used by scientists to describe the tools bacteria use to attack their hosts. For *P. gingivalis*, these factors allow for the colonization of the oral cavity, destruction of periodontal tissues, and evasion of the host's immune system. These tools consist of various molecules and structures developed by the bacteria, many of which are found on its surface.

The first of these tools is the bacterium's outermost layer, known as the capsule. This slimy structure, com-

Diagram illustrating the key steps in determining atomic structure using cryogenic microscopy



MARIUSZ MADEJ, IMAGES OBTAINED USING THE SOFTWARE CRYOSPARC AND PYMOL

A. During the sample analysis, thousands of images of grids with embedded proteins are recorded. In this case, the secretion system for lipoproteins described in the article was captured during fimbriae secretion. For this specific sample, the fimbriae structure can be determined, while analyzing the secretion system itself requires further optimization of conditions.
B. The subsequent stages require specialized software and high-speed computing resources available in Poland via the PLGrid platform. This platform facilitates the selection of specific molecules to analyze, in this case, fimbriae.
C. Based on the two-dimensional images, the software generates a three-dimensional reconstruction.
D. If the data quality is sufficient, further optimization steps allow an atomic model of the molecule to be created, revealing various structural details. At the highest resolution, the positions of individual atoms can be identified, while at lower resolutions, the overall molecular shape is visible.

posed primarily of sugar molecules, surrounds the cell and shields it from environmental threats. The capsule also makes it more difficult for the immune system to detect the bacteria. Its presence not only hinders the pathogen's removal from the body but also supports the formation of biofilms. Another important structural element is lipopolysaccharide (LPS), a molecule that is part of the outer membrane of Gram-negative bacteria. LPS is essential for maintaining membrane stability and also stimulates the immune system, often triggering inflammation.

Other critical virulence factors include protein-cleaving enzymes known as *proteases*. These enzymes break down host proteins into smaller fragments called peptides. The most well-studied proteases produced by *P. gingivalis* are gingipains. Located on the bacterial surface, though they can also be secreted into the surrounding environment, gingipains degrade proteins of the host's immune system. They also likely play a key role in the neurotoxic effects of this pathogen. Research, including studies from our laboratory, suggests that gingipains can digest proteins within nerve cells, leading to their damage. This neurotoxic effect has been observed in Alzheimer's disease.

The bacterial surface also features hair-like structures called *fimbriae*. These components function as adhesive tools, allowing the bacteria to bind to various molecules in the oral cavity – imagine them as sticky threads. They attach to proteins in gingival pockets and to the surface of cells in tissues surrounding the teeth. Additionally, fimbriae can interact with immune system cells, potentially activating them. Research on mice suggests that inflammation triggered by these bacterial structures can lead to the degradation of alveolar bone, which is the structure that supports tooth roots. Fimbriae also activate cells lining blood vessel walls, potentially contributing to the formation of atherosclerotic plaques and the development of vascular atherosclerosis. Furthermore, these structures facilitate the aggregation of *P. gingivalis* with other bacteria, enabling the exchange of signaling molecules or growth-promoting substances. Fimbriae also help the bacteria adhere to solid surfaces, making them crucial for the formation of both initial and mature biofilms.

Although the molecular structure and key pathogenic functions of fimbriae are well understood, the processes involved in their secretion and exposure on the bacterial surface remain unclear. Fimbrial molecules are produced inside the bacteria and cannot pass through the outer membrane on their own. A specialized protein system must exist to facilitate their secretion. Understanding this system in detail could help develop compounds to inhibit the process, thereby reducing the pathogenicity of *P. gingivalis*.

A mysterious mechanism

The fimbriae are made up of numerous components (protein subunits) known as fimbrillins. These subunits are primarily derived from lipoproteins – proteins bound to lipid (fat) molecules. Lipoproteins are common in Gram-negative bacteria and are synthesized in the cytoplasm. Each lipoprotein molecule includes a signal peptide – a short sequence that provides instructions on where the molecule should be transported, whether to a specific location within the cell or outside of it.

Specialized transport systems move these molecules across the inner membrane into the periplasm. There, a specific enzyme removes the signal peptide, and three lipid chains are attached to the newly

exposed end of the protein. These lipids act as anchors, securing the protein to membranes. Some lipoproteins embed themselves in bacterial membranes inside the cell, while others, like the precursors of *P. gingivalis* fimbrial subunits, are transported across the outer membrane. These proteins are known as *surface lipoproteins* due to their final location.

The mechanisms underlying the final stage of protein transport, known as secretion, are highly varied and have been described for relatively few molecules. Typically, secretion involves the formation of a specialized protein channel (secretion system), which functions like a tube, allowing specific proteins to be transported outside the cell. However, a secretion system for fimbriae has yet to be identified in *P. gingivalis*. This system is likely critical for the bacterium, as *P. gingivalis* also produces many other surface lipoproteins that play essential roles, such as in nutrient and heme acquisition, which are vital for its survival.

In our lab, we have identified the proteins that make up the system responsible for secreting lipoproteins and displaying them on the cell surface. This discovery was made by observing that *P. gingivalis* mutants lacking the proteins involved in this system were unable to produce fimbriae. Based on our findings, the system consists of a protein that forms a channel in the outer membrane and an interacting protein located in the periplasm. The periplasmic protein resembles chaperone proteins, which assist other proteins in maintaining their proper shape. Bioinformatic analysis revealed that similar proteins are present in other closely related bacteria. This suggests that the mechanism we are studying may be universal and, therefore, important for many microorganisms.

Attempting to solve the mystery

The first step in our research is to study the three-dimensional structures of the proteins that make up the transport system. Like all proteins, these are chains composed of amino acid residues, which serve as their building blocks. The way these chains fold into specific shapes in three-dimensional space determines the protein's function – much like the shape of a tool determines its use.

To determine the structures of these proteins, we will use two leading structural biology techniques: cryo-electron microscopy (cryo-EM) and X-ray crystallography. In cryo-EM, purified proteins are frozen at extremely low temperatures on specialized grids. This preserves their natural spatial arrangement (conformation), enabling the study of conformational changes. An electron microscope captures thousands of images of protein molecules from different angles, and by combining this data, we can reconstruct the protein's three-dimensional structure.

X-ray crystallography takes a different approach. It requires first obtaining protein crystals, effectively trapping the molecules in a crystalline structure – a process that is often difficult and time-consuming. Once the crystals are obtained, they are then exposed to X-rays, which diffract as they interact with the crystal. The resulting diffraction patterns depend on the angle of the X-ray beam and the arrangement of electron clouds around the atoms in the protein. These patterns are recorded and used to generate an electron density map, showing regions of electron concentration. From this, the positions of individual atoms can be determined, enabling the construction of a detailed three-dimensional model of the crystallized protein.

Each of the two methods has its own limitations. Cryo-EM captures the dynamic behavior of proteins, but it is less effective for studying small proteins and generally offers lower resolution compared to X-ray crystallography. X-ray crystallography, by contrast, provides much higher resolution but only reveals the static arrangement of the protein in its crystal form, offering no insight into its natural dynamics. Moreover, the crystalized state may not fully represent the protein's natural structure. Combining both methods allows for the most comprehensive analysis, yielding detailed and complementary insights into the proteins' structures.

In the later stages of this project, we plan to identify other proteins involved in transporting lipoproteins to the cell surface. We will analyze proteins that interact with the transport system temporarily and determine the amino acid sequence of the signal peptide mentioned earlier. Knowing this sequence will enable us to identify additional surface lipoproteins in these bacteria. This is particularly important because surface-localized proteins are excellent candidates for vaccine development. In an era of increasing antibiotic resistance, such vaccines could offer a promising strategy for preventing infections and reducing their impact.

This research is conducted as part of a project funded by the National Science Centre, Poland (Project No. UMO-2023/51/D/NZ1/02675), led by Dr. Mariusz Madej.

Part of this publication was developed under the initiative of the Polish Minister of Education and Science, "Support for conducting scientific research and development work using the research infrastructure of the National Synchrotron Radiation Centre SOLARIS," based on Agreement No. 1/SOL/2021/2. The research presented in this work was performed at the SOLARIS Centre using the cryo-electron microscope. We would like to thank Michał Rawski, Grzegorz Ważny, and Marcin Jaciuk for their support.

We express our sincere gratitude to the Polish High-Performance Computing Infrastructure PLGrid (HPC Centre: ACK Cyfronet AGH) for providing computational resources and support under the computational grant No. PLG/2024/017163.

Further reading:

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